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Deficiency of *CRTAP* in non-lethal recessive osteogenesis imperfecta reduces collagen deposition into matrix

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Abstract

Deficiency of any component of the ER-resident collagen prolyl 3-hydroxylation complex causes recessive osteogenesis imperfecta (OI). The complex modifies the α1(I)Pro986 residue and contains cartilage-associated protein (CRTAP), prolyl 3-hydroxylase 1 (P3H1) and cyclophilin B (CyPB). Fibroblasts normally secrete about 10% of CRTAP. Most *CRTAP* mutations cause a null allele and lethal type VII OI. We identified a 7-year-old Egyptian boy with non-lethal type VII OI and investigated the effects of his null *CRTAP* mutation on collagen biochemistry, the prolyl 3-hydroxylation complex, and collagen in extracellular matrix. The proband is homozygous for an insertion/deletion in *CRTAP* (c.118_133del16insTACCC). His dermal fibroblasts synthesize fully overmodified type I collagen, and 3-hydroxylate only 5% of α1(I)Pro986. *CRTAP* transcripts are 10% of control. CRTAP protein is absent from proband cells, with residual P3H1 and normal CyPB levels. Dermal collagen fibril diameters are significantly increased. By immunofluorescence of long-term cultures, we identified a severe deficiency (10–15% of control) of collagen deposited in extracellular matrix, with disorganization of the minimal fibrillar network. Quantitative pulse-chase experiments corroborate deficiency of matrix deposition, rather than increased matrix turnover. We conclude that defects of extracellular matrix, as well as intracellular defects in collagen modification, contribute to the pathology of type VII OI.

Keywords

cartilage-associated protein; collagen; matrix insufficiency; osteogenesis imperfecta

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Conflict of interest There are no conflicts of interest relevant to this article.

Supporting Information The following Supporting information is available for this article: Table S1. Effect of *CRTAP* mutation on expression of the 3-hydroxylation complex.
Appendix S1. Detailed case report
Additional Supporting information may be found in the online version of this article.
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Osteogenesis imperfecta (OI) is a heterogeneous heritable connective tissue disorder characterized by bone fragility and deformity. The majority of OI cases have dominant inheritance (Sillence types I to IV OI) and result from mutations in the *COL1A1* or *COL1A2* genes, encoding the proα1(I) and proα2(I) chains of type I collagen, the major structural protein of bone (1, 2). Biochemically, collagen structural defects delay helical folding, exposing the chains to post-translational prolyl 4-hydroxylation and lysyl hydroxylation for a longer time, resulting in 'over-modification' and delayed electrophoretic migration of collagen chains.

In the last 5 years, a few recessive forms of OI have been shown to be caused by defects in the genes encoding the components of the collagen prolyl 3-hydroxylation complex (3, 4): cartilage-associated protein (*CRTAP*) (type VII OI, OMIM #610682) (5, 6), *LEPRE1* (7–9) (type VIII OI, OMIM #610915), and *PPIB* (10–12) (type IX OI, OMIM #259440). Recently, additional disease loci responsible for recessive OI have been identified: FKBP10 (13), SERPINH1 (14), SP7/OX (15), and SERPINF1 (16). While both FKBP10 and SERPINH1 code for collagen chaperones resident in the ER, products of the latter two genes instead are not directly involved in collagen production or secretion but are key factors in osteoblasts differentiation and activity.

Patients with defects in the components of the ER-resident 3-hydroxylation complex have moderate to severe/lethal OI, with white sclerae, small to normal head circumference and structurally normal collagen. Loss-of-function mutations in *CRTAP* and *LEPRE1* result in rhizomelia, decreased to absent 3-hydroxylation of α1(I)Pro986, and collagen helical overmodification indicative of delayed folding. Of the three components of the 3-hydroxylation complex, CRTAP is known to be secreted into the extracellular matrix (17, 18). Normally, about 10% of CRTAP is secreted, while most is retained in the ER in a complex with prolyl 3-hydroxylase 1 (P3H1).

Sixteen *CRTAP* mutant alleles, occurring in 15 index probands, have been reported (5–7, 18–20). Most null cases are lethal in the perinatal period or within the first year of life. Five non-lethal cases have been described. We present here a 7-year-old Egyptian boy whose severe OI is caused by homozygosity for a frameshift mutation in *CRTAP* exon 1. His dermal fibroblast type I collagen has typical post-translational modification defects for type VII OI. We report here the novel finding that the collagen content of matrix deposited by patient cells in culture is severely decreased. This data is supported by an *in vitro* collagen matrix-chase assay. These investigations describe matrix deficiency and disorganization associated with CRTAP deficiency which may reflect the absence of the crucial functions of CRTAP in extracellular matrix.

Materials and methods

For details of all methods see Appendix S1, Supporting information.

Results

Clinical summary

The proband (patient 903) is a 7-year-old Egyptian boy who was ascertained in Verona when his parents emigrated to Italy. He was the product of a term gestation whose skeletal dysplasia was detected on prenatal ultrasound. At birth, weight and length were normal but head circumference was fifth percentile. Nasal CPAP was required for 1 week after birth.

Currently, he has severe OI, with grayish sclerae, vertebral deformities, rhizomelia and fractures and deformities of long bones. He also has a history of recurrent obstructive bronchiolitis. He has been treated with intravenous neridronate since 3 months of age and currently has a DXA z-score of −3 SD. At age 6 years, he had pronounced growth deficiency with the length of an average 18-month-old boy and a head circumference average for his age. His gross motor function is limited to standing with assistance. Representative radiographs show limb deformities, and platyspondyly of cervical, dorsal and lumbar vertebral bodies (Fig. 1a–e). The detailed case report is presented in Appendix S1.

Mutation detection

Direct sequencing of patient with *COL1A1* and *COL1A2* genes yielded negative results. Sequencing of genes causing recessive OI revealed that the patient is homozygous for an insertion/deletion mutation (c.118_133del16insTACCC) in exon 1 of *CRTAP* (Fig. 1f). This mutation shifts the *CRTAP* reading frame, leading to a premature termination codon, 117 codons downstream of the mutation in exon 2. The *Mwo*I restriction site introduced by the mutation was utilized to confirm homozygosity in the patient and heterozygosity in both parents (Fig. 1g).

Expression of genes coding for collagen prolyl 3-hydroxylation complex

Transcript levels for the genes coding for components of the collagen 3-hydroxylation complex were measured in cultured patient fibroblasts by real-time RT-PCR. *CRTAP* transcript levels were normalized to three reference genes; patient cells had 10% expression of control cells (Table S1, Supporting information), confirming that the mutation led to a null allele.

CRTAP and P3H1 proteins are absent from patient cells

Western analysis using antibody to residues 307–401 in the C-terminus of CRTAP was unable to detect protein in patient fibroblasts (Fig. 2a). Because CRTAP and P3H1 are mutually protective (14), P3H1 levels were barely detectable on Westerns, while levels of CyPB were normal. Examination of patient cells by immunofluorescence corroborated absence of both CRTAP and P3H1 from the ER (Fig. 2b).

Collagen post-translational modification

The type I collagen chains of the patient migrated as broad bands on SDS-Urea PAGE, consistent with the full overmodification previously described in collagen secreted by cells with *CRTAP* mutations (Fig. 2c) (5). Collagen from both media and cell layer fractions were electrophoretically delayed; overmodified forms were not retained in cells. In addition, α1(V) chains were slightly reduced in quantity and had delayed electrophoretic migration.

The proportion of helical hydroxylysine/total lysine was 40% (normal 19–25%), confirming full overmodification of the collagen helix. As expected of overmodified collagen with a normal primary sequence, the T_m of patient collagen was increased about 1°C (Fig. 2d). 3-hydroxylation of α1(I)P986 in patients was barely detectable (5% *vs* normal 95–98%), as expected from a *CRTAP* null allele.

Reduced collagen content of matrix deposited in culture

Type I collagen deposited in extracellular matrix by long-term cultures of control and OI fibroblasts was analyzed by indirect immunofluorescence using a polyclonal antibody to type I collagen. In control fibroblasts, a regular fibrillar network surrounding the cells was evident, whereas in OI cells, type I collagen was severely reduced and appeared poorly organized with a barely visible fibrillar network (Fig. 3a).

A pulse-chase study was done to confirm that this reduction in matrix was a defect in deposition rather than matrix turnover. Patient fibroblast cultures maintained for 2 weeks post-confluence deposited approximately 10% the amount of type I collagen (α1(I): 6%; α2(I): 12%) as did control cells (Fig. 3b,c), corroborating the immunofluorescence studies. When the pulse of deposited collagen was chased for 5 days, loss of collagen from matrix was not accelerated in patient compared to control.

Dermal collagen fibrils have increased diameter

Dermal collagen fibrils of the patient were compared to an age-matched control by electron microscopy. The diameters of patient fibrils were larger than control fibrils (patient 92.2 ± 8.9 nm; control 89.0 ± 6.4 nm, $p < 0.001$ using Student's *t*-test; Fig. 1h). Patient fibril diameters also displayed significantly greater variability than control fibrils ($p < 0.001$, using *F*-test).

Discussion

The patient presented in this report is a 7-year-old Egyptian boy with severe non-lethal recessive type VII OI, caused by a homozygous null mutation in *CRTAP*. Typical of recessive OI caused by defects in the components of the collagen prolyl 3-hydroxylation complex, the patient has light sclerae, a normal head circumference, rhizomelia and extreme short stature. The severity of his osteochondrodysplasia is comparable to dominant OI type III. At the molecular level, our patient has a *CRTAP* null mutation, resulting in reduction of *CRTAP* transcript levels to approximately 10% of normal levels, and undetectable CRTAP protein in fibroblasts. The abnormal post-translational modification of his type I collagen is also typical for type VII OI, with α1(I)Pro986 3-hydroxylation reduced to 5% of normal, and full helical overmodification indicated by 40% hydroxylysine levels.

Our patient is now the sixth proband with a nonlethal *CRTAP* mutation, of 16 reported cases. A hypomorphic mutation in intron 1 (c.472-1021C>G) in a First Nations pedigree that first defined type VII OI is moderately severe (6). Baldridge et al. (7) reported two severe non-lethal cases, a 9-year-old Iranian girl who was homozygous for a missense mutation in exon 1 (c.200T>C; p.Leu67Pro) and a 1-year-old Caucasian girl, who was a compound heterozygote with frameshift mutations in exons 1 and 4. Van Dijk et al. (20) also reported two severe non-lethal Caucasian children, a 2-year-old girl with a frameshift mutation in *CRTAP* exon 1 (c.21_22dupGG; p.Ala8fsX), and a 4-year-old boy with a c.471+2C>A defect in intron 1. Although our patient was treated with bisphosphonate since infancy, the other five non-lethal cases did not have pharmacological intervention, indicating that bisphosphonate treatment is not the critical factor in survival. Levels of *CRTAP* transcripts and protein are not distinctive between lethal and non-lethal cases. *CRTAP* transcript levels have been determined in nine cases, revealing normal levels in the non-lethal missense mutation (7), but 4–10% of normal transcripts in null non-lethal cases, which overlaps with 1–25% of normal levels in lethal cases (5–7, 18). Residual CRTAP protein has been detected only in cells with the hypomorphic First Nations mutation, where an intronic mutation leads to a low level of normal transcripts, but not in null lethal mutations (5, 6, 18). Our case is the first non-lethal CRTAP mutation studied by Western blot. CRTAP is undetectable in our patient, as in the lethal cases; however, the nonspecific binding of antibodies to the N-terminal end of CRTAP precludes our exclusion of residual truncated protein. Similarly, examination of the eight cases in which the level of Pro986 hydroxylation has been quantitated reveals 79% 3-hydroxylation in collagen from the cells with a *CRTAP* missense mutation, but 5–21% 3-hydroxylation among other non-lethal cases, which overlaps the 4–40% 3-hydroxylation among lethal cases (5–7). Furthermore, all reported *CRTAP* mutations except one are located in exon/intron 1 or exon 4, which may represent

regions crucial for mutual stabilization with P3H1. However, non-lethal cases do not have a preferential location within the gene (5–7, 18–20). Other factors related to the mechanism of type VII OI, either in terms of ER stress or abnormal structure of collagen fibrils in matrix, are thus implied to contribute to the lethality of the condition.

Complementary long-term cultures and pulse-chase studies conducted with our patient's fibroblasts demonstrated the novel finding of severe deficiency of type I collagen deposition in extracellular matrix. Total collagen secretion from cells is essentially normal, however, as is turnover of the deposited collagen. The severe collagen matrix deficiency is unlikely to be related to the abnormal post-translational modification of the collagen helix, since it has not been seen as a consistent feature of dominant OI. It is more likely to be related to the absence of CRTAP from matrix. CRTAP is a secreted molecule and its role in extracellular matrix is unknown. Finally, the matrix deficiency and disorganization may be the extracellular effect of the diminished prolyl 3-hydroxylation. Weis et al. (21) proposed a role for collagen prolyl 3-hydroxylation in the supramolecular assembly of molecules in a D-period staggered array, consistent with our findings. In this potential mechanism, the matrix disorganization may be related to absence of 3-hydroxylation of type I as well as type V collagen, since the ER-resident 3-hydroxylation complex also hydroxylates α2(V)Pro986 (21). As the polymerization of type I fibrils requires type V collagen as template (22, 23), the concomitant loss of 3-hydroxylation at α2(V)Pro986 could contribute to both our findings and the severity of the phenotype. In this regard, it is interesting to note the slight decrease in quantity and overmodification of α1(V) in the collagen screening as well as the clinical signs of our patient (joint laxity, inguinal hernia, and soft skin) which overlap in OI and EDS phenotypes. These findings will need to be confirmed in other cases before they can be confidentially related to phenotype. However, the intriguing results from this single proband direct our future investigations on the mechanism of types VII and VIII OI on types I and V collagen to matrix, as well as to collagen post-translational modification.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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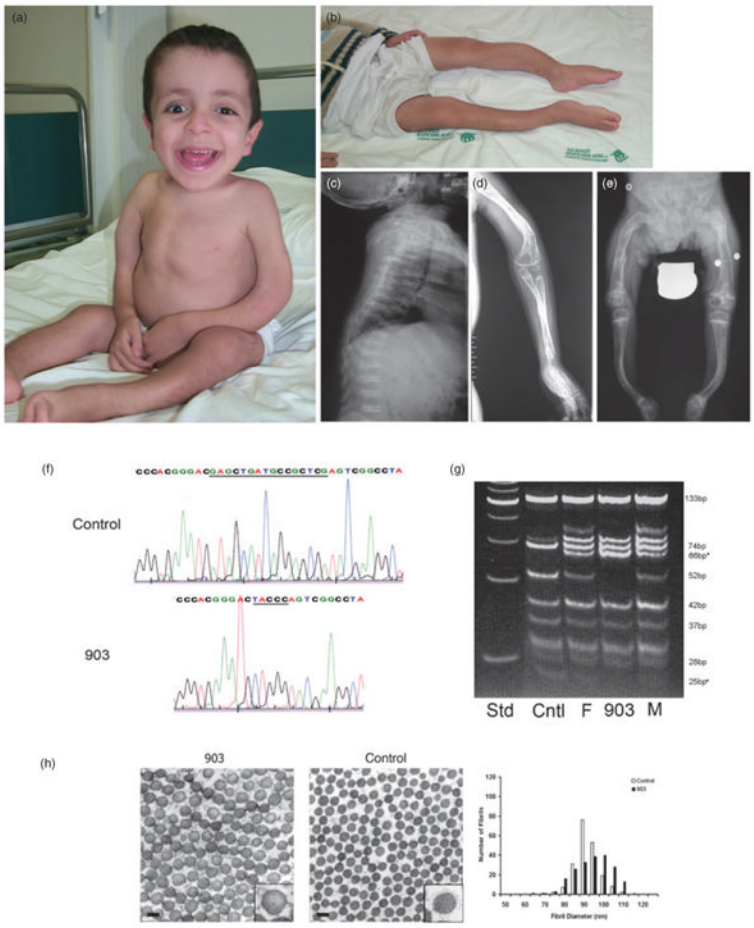

Fig. 1.

Patient 903 features, mutation identification, and fibril diameters (**a**) At 5 years, 10 months, the patient has a normal head, white sclerae, small thorax and shortening of the proximal segment of the upper and lower extremities. (**b**) Detail of short bowed legs. Radiographs of: (**c**) spine, (**d**) arm, (**e**) legs show osteopenia, undertubulation and severe deformities, consistent with a severe deforming form of osteogenesis imperfecta. (**f**) Sequence tracing of patient 903 gDNA shows a homozygous insertion/deletion in exon 1 of *CRTAP*. The mutation deletes the 16 nucleotides underlined in the control sequence and inserts the five nucleotides shown in the patient sequence. (**g**) A *Mwo*I restriction enzyme digest of patient 903 gDNA and parental gDNA confirms the presence of the homozygous mutation in the patient and heterozygosity of the mutation in his father (F) and mother (M), respectively. (**h**) Patient dermal collagen fibrils from a skin punch biopsy were compared to an age-matched control and examined by transmission electron microscopy. The fibril diameters ($n = 200$) were increased, and fibrils had slightly irregular borders and increased size variability.

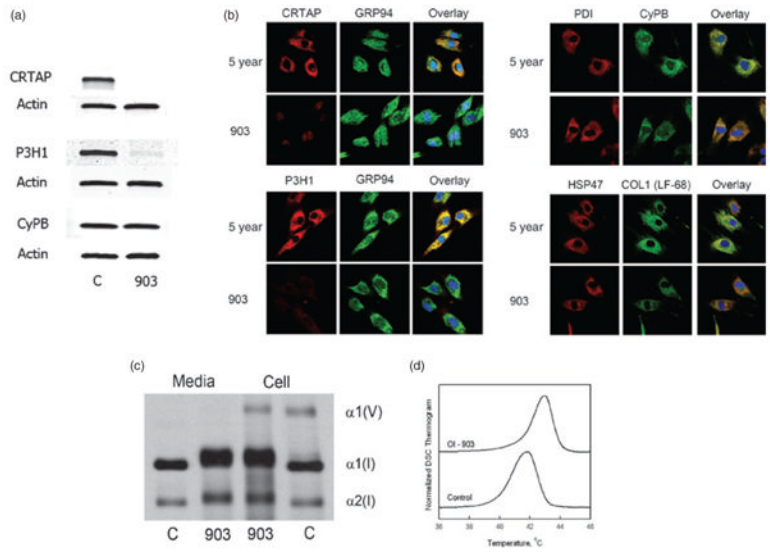

Fig. 2.

Effects of *CRTAP* mutation on the prolyl 3-hydroxylation complex and on type I collagen. (**a**) Western blots of cartilage-associated (CRTAP), prolyl 3-hydroxylase 1 (P3H1) and cyclophilin B (CyPB) protein levels in patient 903 fibroblasts compared to actin-loading controls. Due to mutual protection, the absence of CRTAP leads to the loss of P3H1. CyPB levels remain normal. (**b**) Immunofluorescence staining of fibroblasts of patient 903 and control for the three components of the prolyl 3-hydroxylation complex, CRTAP (top left), P3H1 (bottom left), CyPB (top right), plus type I collagen (bottom right), shown colocalized with the endoplasmic reticulum chaperones GRP94, PDI or HSP47. The immunofluorescence confirms the lack of CRTAP and P3H1 in fibroblasts. (**c**) Procollagen synthesized during metabolic labeling with tritiated proline by control (C) and patient (903) was partially purified from medium (M) and cell layer (CL), pepsin digested, and analyzed on 6% SDS-Urea PAGE. Alpha chains in medium and cell layer have a delayed migration and appear overmodified. No normal migrating chains are evident. (**d**) A differential scanning calorimetry thermogram shows that the collagen melting temperature is increased by ~1°C, consistent with the increased collagen modification.

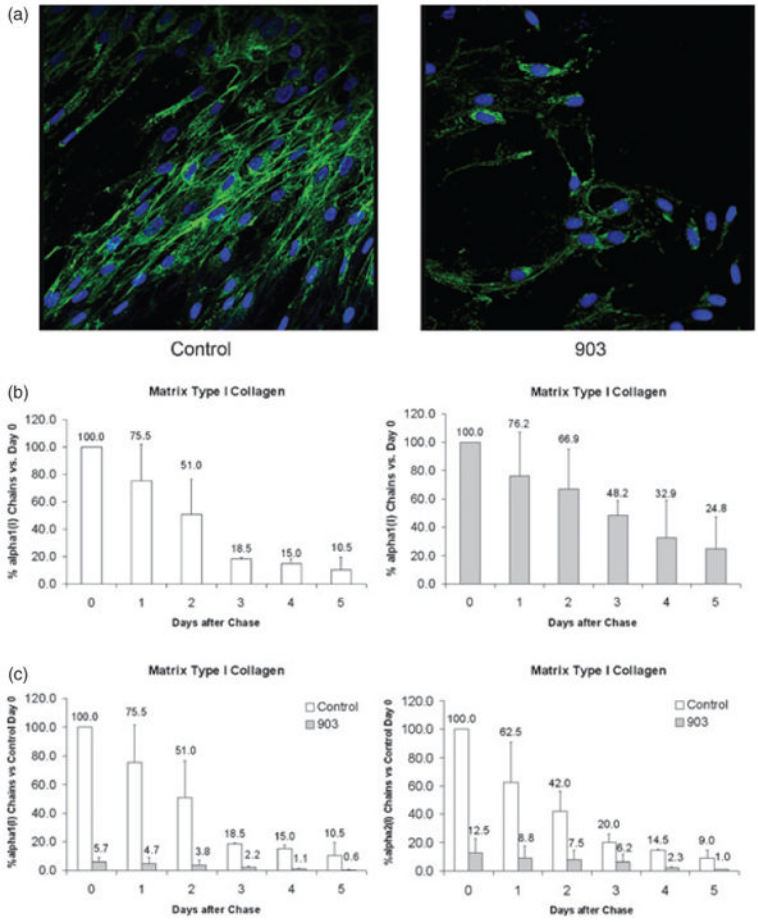

Fig. 3.

Decreased collagen deposition in ECM after long-term culture. (**a**) Control and patient fibroblasts were maintained for 21 days after confluence. After medium removal, the cell layers were labeled with a polyclonal anti-type I collagen, LF-67, and nuclei were counterstained with DAPI. The fibrillar network is absent in patient 903. (**b**) Quantitation of collagen turnover in matrix, with the day 0 time point arbitrarily set to 100, showing similar rates of turnover between control (white) and patient cells (gray). (**c**) Comparison of α1(I) chain (left) and α2(I) chain (right) deposition and turnover of control and patient matrix, showing much less matrix deposited from patient 903 cells.